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PRINCIPAL INVESTIGATOR: Mina J. Bissell, Ph.D.

CONTRACTING ORGANIZATION: University of California Berkeley
Berkeley, CA 94720

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Mina J. Bissell, Ph.D.

E-Mail: MJBissell@lbl.gov

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14. ABSTRACT: Recent studies have demonstrated that breast tumors contain highly tumorigenic progenitor subsets of cells that may be the primary contributors to progression, metastasis and resistance to therapy. Preliminary data using a highly tumorigenic human breast cancer cell line model that resembles these progenitor subpopulations demonstrate that these cells internalize metal-labelled hyaluronan (HA) nanoparticles with a greater capacity than breast tumor cell lines that do not exhibit progenitor breast tumor cell characteristics. Award authorization in this first grant year was significantly postponed, causing a delay in our progress. However we have succeeded in confirming this preliminary data by showing increased uptake of HA by MDA-MB-231 cells as compared to MCF- 7 cells and by transformed mouse fibroblast cells as compared to their non-transformed counterparts, and have identified the HA receptor required for HA uptake, CD44. These are important first steps towards validating the utility of HA-metal nanoparticles for the isolation and imaging of discrete highly tumorigenic subpopulations. These studies may provide insight into the subsets of cells in human tumors that may play a key role in the progression of breast cancer and could also lead to better techniques for imaging breast tumors in patients.

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INTRODUCTION

Despite improvements in the treatment and early diagnosis of breast cancer, this disease represents a major cause of cancer related deaths in women. Recent studies have demonstrated that breast tumors contain highly tumorigenic progenitor subsets of tumor cells that may be the primary contributors to progression, metastasis and resistance to therapy. These progenitor cell subpopulations have been partially characterized and sorted by fluorescent activated cell sorting based on the expression of specific cell surface markers. Preliminary data using a model of a highly tumorigenic human breast cancer cell line that resembles these progenitor subpopulations demonstrate that these cells can also internalize metal-labelled hyaluronan (HA) nanoparticles with a greater capacity than breast tumor cell lines that do not exhibit characteristics of the progenitor breast tumor cells. HA internalization was found to be dependent on the function of CD44 and Rhamm, which are two HA receptors associated with breast tumor invasion and progression. HA is an extracellular matrix polysaccharide that accumulates in malignant tumors. HA is also associated with tumor progression. The purpose of this proposal is to determine the potential utility of HA-metal nanoparticles for isolating and imaging discrete highly tumorigenic subpopulations. Experiments are proposed using defined xenograft injection models to determine the efficacy of using labeled HA conjugates for tumor imaging *in vivo*. Experiments are also proposed using human tumor samples to evaluate the potential of this HA-metal nanoparticle technology for identifying highly tumorigenic subsets in clinical isolates of advanced primary breast cancer. These studies have the potential to provide new approaches for the isolation and characterization of the tumorigenic subsets in human tumors that may play a key role in the progression of breast cancer. Studies also could lead to better techniques for imaging breast tumors in patients, which could improve the staging of recently diagnosed breast tumors. This could facilitate better therapeutic approaches, especially in newly diagnosed patient subgroups with an uncertain prognosis.

BODY

Due to outstanding human subjects and animal use protocol approvals at the DOD level, this award was only opened to us on March 16, 2006. Prior to that date, LBNL would not authorize the award due to their understanding from DOD that no research could be performed until we had received approvals of all protocols. Although our human subjects approval is currently still pending (it is currently undergoing second-level review by Ms. Jo Collins; our animal use protocols were recently approved), LBNL recently came to an agreement with DOD that the award could be opened with restrictions that no human subjects research could be performed until approval.

Since the award was only open to us approximately a month ago, we have not been able to make the progress on this proposal as we originally described in our Statement of Work. We do intend to complete the work we proposed and will apply for a 1 year No Cost Extension to enable us to do so. The following describes the work that has been accomplished in the past month and our plans for the coming award year.

In spite of the delay in award authorization, we have been able to make some quick progress in the past month regarding Task 1. We first confirmed that labeled hyaluronan is taken up into cultured tumor cells. For these experiments we initially prepared Texas Red-hyaluronan conjugates and assessed uptake using confocal microscopy and image analysis (Simple PCI) software. The method for linking Texas Red to hyaluronan is shown in Figure 1A. An example of the uptake of this conjugate into cultured cells is also shown in Figure 1B. The area of the cell used to quantify uptake of the hyaluronan conjugate is shown as a dotted line. Using this method, we next compared uptake of Texas Red-hyaluronan in mouse fibroblast tumor lines (ras-transfected, RHAMM-transfected) with their non-transformed parental counterpart. As shown in Figure 1B, the tumor lines incorporated Texas Red-hyaluronan to a greater extent than the parental fibroblast line. Similarly, we compared uptake of hyaluronan in MDA-MB-231 breast tumor cells, used as an example of an aggressive breast cancer line that exhibits tumor progenitor properties (Wang et al., 2002; Al-Hajj et al., 2003), with MCF-7 breast tumor cells that are not as aggressive and do not exhibit similar tumor progenitor properties as the MDA-MB-231 cells. As shown in Figure 1C, the more aggressive MDA-MB-231 tumor cells incorporated Texas Red-hyaluronan to a greater extent than MCF-7 tumor cells. Growth in monolayer culture does not accurately replicate the microenvironment that tumor cells encounter *in vivo*. Therefore, we next grew the MDA-MB-231 and MCF-7 breast tumor cells as 3 dimensional aggregates in collagen gels, which mimic aspects of the *in vivo* microenvironment. For these assays, we incubated tumor cell aggregates with gadolinium-decorated-hyaluronan (Gouin and Winnik. 2001), released the aggregates from collagen gels, washed them and then quantified uptake of gadolinium-hyaluronan with MRI. As shown in Figure 1D, MDA-MB-231 cell aggregates (or pellets) consistently took up greater amounts of gadolinium-hyaluronan than MCF-7 aggregates. These results thus confirm preliminary data provided in the original grant application.

Breast tumor progenitor subsets have been reported to express high levels of the hyaluronan receptor, CD44 (Al-Hajj et al., 2003; Ponti et al., 2005). We have predicted that progenitor cells will rapidly take up labeled hyaluronan as a result of their high CD44 expression. Therefore we expect that function blocking anti-CD44 antibodies will reduce labeled hyaluronan uptake. For these experiments, we assessed the uptake of Texas Red-hyaluronan in CD44+ MDA-MB-231 tumor cells in the presence of non-immune IgG or two anti-CD44 antibodies, KM114 and KM201. As shown in Figure 2, the anti-CD44 antibody KM201 significantly blocked uptake of Texas Red-hyaluronan while KM114 blocked uptake to a lesser extent. As KM201 is known to block hyaluronan interaction with CD44 more effectively than KM114, these results are consistent. These results show that CD44 is required for uptake of hyaluronan in MDA-MB-231 and likely other breast tumor progenitor cell lines as well.

Plans for the upcoming year include further work on Task 1, that is, performing similar experiments as the ones depicted in Figure 1A-C, but comparing an E-cadherin transfected "revertant" MDA-MB-231 cell lines with its parental MDA-MB-231 parental cell line. We also plan to shortly commence work on Task 2, the development of various GD-HA preparations. We will then assess the uptake of these GD-HA preparations in the E-cadherin transfected MDA-MB-231 and parental cell lines as described in Task 3, similarly as in the experiments described here in Figure 1D. Tasks 1-3 are all described in the Statement of Work to be performed in Year 1 of the award, but as the award was so recently authorized, we plan to complete these tasks in the coming year. If time permits, we will also begin work on Tasks 4 and 5. All other tasks are

currently on hold as they involve human use, which we are not currently authorized to perform until DOD approval.

KEY RESEARCH ACCOMPLISHMENTS

- Confirmation of preliminary results showing that aggressive cell lines with tumor progenitor properties incorporate hyaluronan to a greater extent than less aggressive cell lines.
- Determination of the HA receptor required for HA uptake, CD44, which is high in breast tumor progenitor cell lines.

REPORTABLE OUTCOMES

- Funding applied for: Department of Defense (DOD) Fiscal Year 2006 (FY06) Breast Cancer Research Program (BCRP) Multidisciplinary Postdoctoral Award (W81XWH-06-BCRP-MPA); PI: Mandana Veiseh
- Intellectual property disclosure: Lawrence Berkeley National Laboratory, # IB 2006, "Detection of high tumorigenic stem cell populations."

CONCLUSION

Although our progress has been considerably delayed by the postponement in award authorization, we have already succeeded in confirming preliminary data regarding the increased uptake of HA by the aggressive breast cell line MDA-MB-231 as compared to the less aggressive MCF-7 cell line and have identified the HA receptor required for HA uptake in the MDA-MB-231 cells, CD44. This is an important first step towards using hyaluronan uptake as a tool for achieving our ultimate goal of identifying and imaging breast tumor progenitor cells primary tumors of breast cancer patients.

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Wang, F., Hansen, R.K., Radisky, D., Toneda, T., Barcellos-Hoff, M.H., Petersen, O.W., Turley, E.A. and M.J. Bissell. (2002). "Phenotypic reversion or death of cancer cells by altering signaling pathways in three-dimensional contexts." J Natl Cancer Inst **94**(19): 1494-503.

SUPPORTING DATA

Figure 1: Quantification of Texas Red-hyaluronan uptake into mouse fibroblasts and human breast cancer cell lines.

Figure 2: CD44 is required for uptake of Texas Red-hyaluronan into mouse fibroblast tumors.

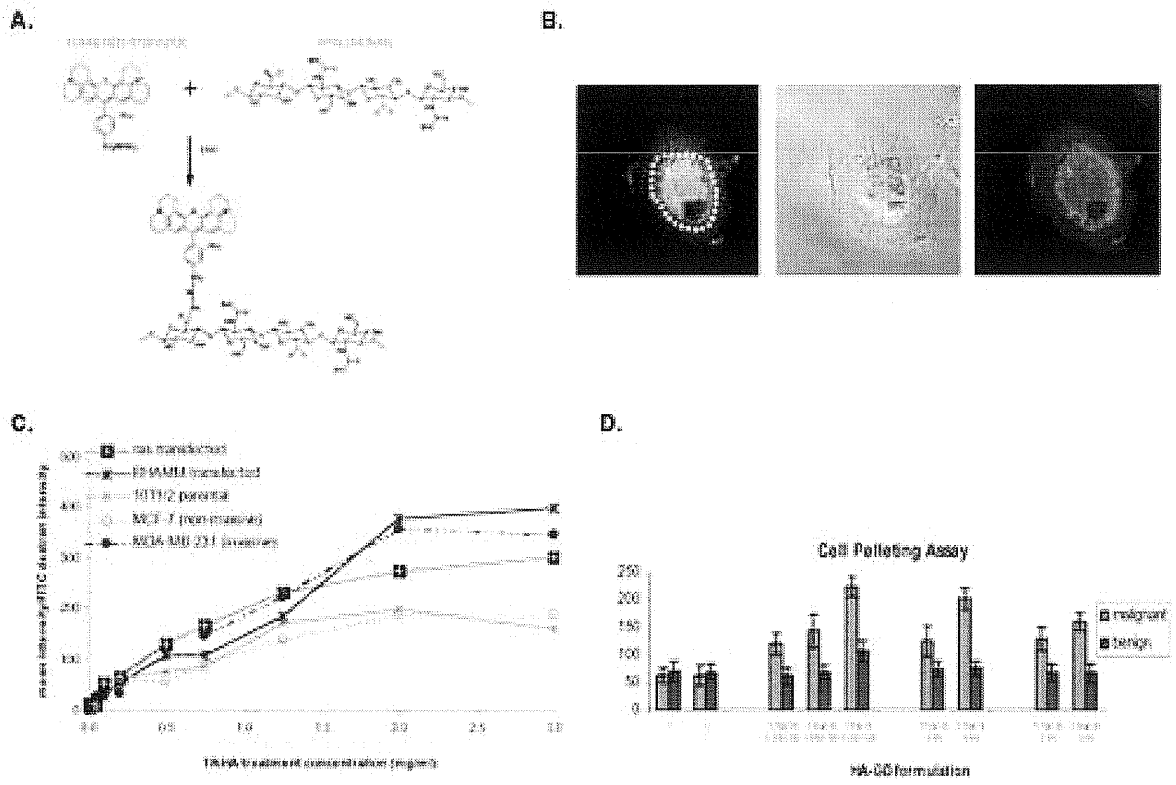
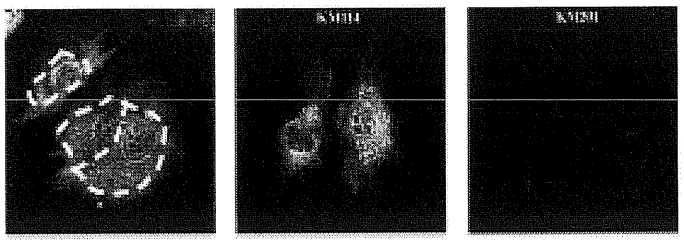


Figure 1. Quantification of Texas Red-hyaluronan uptake into mouse fibroblasts and human breast cancer cell lines. A. Chemistry for preparing Texas Red-hyaluronan. Texas Red hydrazide was reacted with hyaluronan in the presence of EDAC carbodiimide. Reagents were used at dilutions to achieve a 1:10 (Texas Red:hyaluronan disaccharide) stoichiometry. B. Confocal microscopy was performed on cells exposed to the Texas Red-hyaluronan reagent and the circled area was quantified using Simple PCI image analysis software. C. Different concentrations of Texas Red-hyaluronan were added to cultured cell lines and uptake quantified as shown in (B). Aggressive tumor cell lines (Ras-transfected 10T1/2 fibroblasts, RHAMM-transfected 10T1/2 fibroblasts and MDA-MD-231 human breast cancer cells) were confirmed to take up more label than less aggressive (MCF-7 breast cancer cells) or non-transformed cells (parental 10T1/2 fibroblasts). D. Gadolinium-hyaluronan (HA-GD) was added to human breast tumor cell lines grown as aggregates in 3D collagen gels. Aggregates were released from collagen gels, washed and fixed then imaged in MRI. Hyaluronan is taken up to a greater extent in MDA-MB-231 cells (malignant) than in MCF-7 (benign) breast tumor cells.

A.



B.

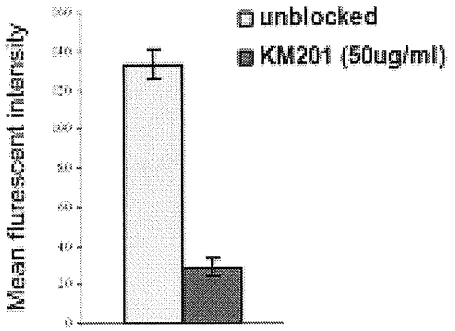


Figure 2. CD44 is required for uptake of Texas Red-hyaluronan into human breast cancer cell lines. A. Confocal microscopy was performed on CD44+ MDA-MB-231cells exposed to the Texas Red-hyaluronan reagent and IgG (left), and the CD44 function-blocking antibodies KM114 (middle), and KM201 (right). KM201 strongly inhibits uptake of Texas Red-hyaluronan into cells while KM114, known to block hyaluronan binding to CD44 less effectively than KM201, does not reduce hyaluronan uptake as efficiently. B. The circled areas were quantified using Simple PCI image analysis software. These results indicate that the hyaluronan receptor, CD44, is required for uptake of tagged hyaluronan into tumor cells.